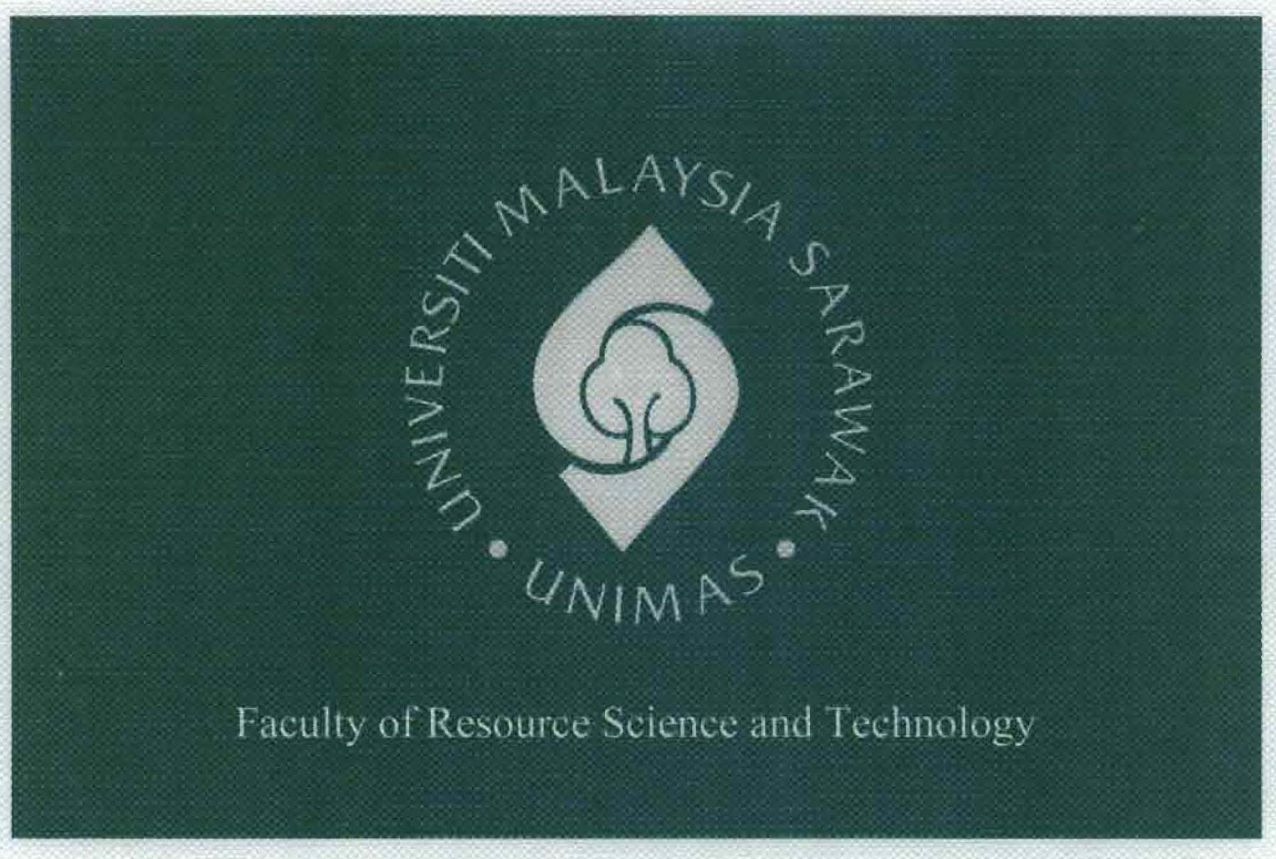

IDENTIFICATION AND CHARACTERISATION OF GUT MICROFLORA OF FRUGIVOROUS BATS FROM SARAWAK

Fatiyah binti Md Kilau

(34841)

Bachelor of Science with Honours
(Resource Biotechnology)
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A Thesis Submitted in Partial Fulfillment of the Requirement of the Degree of Bachelor Science with Honours (Resource Biotechnology)

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DECLARATION

I hearby declare that no portion of the work referred to this thesis has been submitted in support of an application for another degree of qualification to this or any other university or institute of higher learning.

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(Fatiyah binti Md Kilau)

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LIST OF ABBREVATIONS

CFU	Colony forming unit
16S rRNA	16S ribosomal RNA
µl	Microliter
H	Hours
°C	Degree celcius
H_2O_2	Hydrogen peroxide
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
CFU	Colony forming units
$MgCl_2$	Magnesium chloride
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
ddH_2O	Double-distilled water
dNTP	Deoxyribonucleotide triphosphates
pH	potential Hydrogen
NCBI	National Centre for Biotechnology Information

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ABSTRACT

Information that is carried by microbiota community is complex and valuable. However, this information is very limited and only few studies have reported on this. Gastrointestinal tract of bats composed of complex community of microbes and they are well-known as a natural reservoir of zoonotic diseases. Thus, this study aims to discover the microbe diversity in the gut of frugivorous bats from various localities in Sarawak. Microbes was identified to the genus level by using biochemical test and further verified using 16S rRNA gene sequence analysis to identify up to the species level. Estimation of viable bacteria that are present in the gut of frugivorous bat ranged from 1.65×10^5 to 5.2×10^9 CFU/ml. A total of 13 genera of bacteria were identified from 103 isolates of six species of frugivorous bats. Genera of bacteria identified from the gastrointestinal tract frugivorous bats include *Klebsiella, Bacillus, Proteus, Citrobacter, Escherichia, Serratia, Enterobacter, Pseudomonas, Enterococcus, Vibrio, Shigella, Acinetobacter* and *Staphylococcus*. Most of the identified genera come from *Enterobacteriaceae* family. Thus, this study provided insight in understanding bat biology as a host reservoir and gives additional information on the bacteria involved in the diet of frugivorous bats.

Keywords: Borneo, 16S rRNA, Microorganism, Fruit bat

ABSTRAK

Maklumat yang dijalankan oleh komuniti microbiota adalah sangat kompleks dan berharga. Walau bagaimanapun, maklumat ini adalah sangat terhad dan hanya beberapa kajian telah dilaporkan mengenai perkara ini. Saluran gastrousus kelawar terdiri daripada masyarakat kompleks mikrob dan mereka terkenal sebagai takungan semula jadi penyakit zoonotik. Oleh itu, kajian ini dijalankan bertujuan untuk mencari diversiti mikrob dalam usus kelawar makan buah dari pelbagai lokasi di Sarawak. Tahap genus mikrob dikenal pasti dengan menggunakan ujian biokimia dan pengesahan lanjutan analisis jujukan gen 16S rRNA untuk mengenal pasti hingga ke tahap spesis. Anggaran bakteria yang dapat dilihat di dalam usus kelawar makan buah adalah di antara 1.65×10^5 to 5.2×10^9 CFU/ml. Sebanyak 13 genus bakteria telah dikenal pasti dari 100 pencilan daripada enam spesies kelawar makan buah. Genus bakteria yang dapat dikenal pasti daripada saluran gastrousus kelawar makan buah termasuklah Klebsiella, Bacillus, Proteus, Citrobacter, Escherichia, Serratia, Enterobacter, Pseudomonas, Enterococcus, Vibrio, Shigella, Acinetobacter dan Staphylococcus. Kebanyakan genus yang dikenal pasti berasal dari keluarga Enterobacteriaceae. Oleh itu, maklumat yang diperolehi semula pada kajian ini akan memberikan gambaran dalam memahami biologi kelawar sebagai takungan tuan rumah dan memberikan maklumat tambahan mengenai bakteria yang terlibat dalam diet kelawar makan buah.

Kata kunci: *Borneo, 16S rRNA, mikroorganisma, kelawar makan buah*

1.0 Introduction

High abundance of bats worldwide has served various economic and ecological services to the human and their environment (Kasso and Balakrishnan, 2013). In Southeast Asia, most of the bats have either adopted frugivorous or insectivorous diet. This has largely contributes towards regeneration of forest by facilitating seed dispersal and pollination (Calisher *et al.*, 2006). Study conducted by Fukuda *et al.* (2009) found that megachiropteran species has the ability to transport seed over long distance that is the key in forest regeneration. They also provide great economic advantage in Southeast Asia where country like Thailand earns $230 million per year by pollinating for large-scale crops of durian (Fujita and Tuttle, 1991 as cited in Kasso and Balakrishnan, 2013). Their ability to forage on a great variety of food also explains about the great diversity within mammalian lineage (Ley *et al.*, 2008). This impact has been facilitated primarily by the microbe that helps mammal to explore these foods by breaking down the food resources for digestion.

Therefore, understanding the ecology of microorganism in a host can provide insight on the biology of bats and revealed the potential they contributing to the host. Bat has a very unique immune system, which makes them as a natural reservoir host that can be influenced by their diet and environment where they interact (Calisher *et al.*, 2006). Study on the germ-free animals in 1885 has revealed the importance of microbes in the gastrointestinal tract of host (Hansen *et al.*, 2012). The absence of normal microflora in germ-free animals was associated with the high requirements of vitamin B and K, impaired intestinal structure and morphology, and decreased immune defense compared with the conventional animals (Kannock, 1997 as cited in Kil and Swanson, 2011).

Previously, wide range of microbial species primarily from Family *Enterobacteriaceae* and *Staphylococcaceae* has been documented in fruit bat (Daniel *et al.*, 2013). Microbes that colonize bat's gastrointestinal tract are important in nutritional status of bats (Kil and Swanson, 2011). For example, cellulose-degrading and xylanolytic bacteria were found in *Cynopterus sphinx*, which helps in digesting cellulose in fruit bat that feeds on fruits (Anand *et al.*, 2012). In *C. sphinx*, fruit has becoming an important carbohydrate source for them (Anand *et al.*, 2012).

Diets do affect the evolutionary process of new species of bats. Study done by Ley on 13 orders of mammals highlighted that feeding habit of a host is a fundamental driver that determined microflora relationship between hosts (Ley *et al.*, 2008). Ethanol vapor emit from the ripening fruit suggested to be an appetite stimulant and act as a source of energy to the fruit-eating bat (Sanchez *et al.*, 2004).

Only few researches focus on microbiological diversity in the gastrointestinal tract of bat. Previous studies have been concentrated on the area of taxonomy, diversity and the evolutionary history of the bats. However this information is important to determine the ecological significance of these hosts. Thus, the aim of this research is to identify and characterise the gastrointestinal tract microbiota from frugivorous bat species in Sarawak using anal swab method. Specifically, the project will address the importance of diet in shaping the microbial diversity.

1.1 Objectives

This study was conducted with the following objectives:

1. To identify and characterise bat gut microbiota from frugivorous bat species in Sarawak based on biochemical and molecular analysis.
2. To cross verify species identification using 16S rRNA gene sequence analysis

2.0 Literature Review

2.1 Frugivorous bat as the microbial host

Bat, classified under the order Chiroptera is a unique small mammal with a capability of true flight. Although bats represent about a quarter of all species of mammal in the world in terms of species diversity but not much information is known about them especially on their role as mircrobial host. Bats play crucial role to keep the ecosystem in balance by facilitating seed dispersal, predation on insect and important for pollination (Kasso and Balakrishnan, 2013). Bat species are distributed all over the world especially in the tropics regions. Diversity of bat occurs in abundant in Southeast Asia. Their distribution increases as latitude decreases in the equatorial rain forests of South Africa, South America and Asia (Kingston *et al.*, 2003).

There are about 1200 species of bats in the world where fruit bat contributes about 30% of the population. Frugivorous bat can be in contact with human because of their foraging activity (Daniel *et al.*, 2013). They depend on the sense of sight and smell to navigate and find food. This characteristic differed fruit bat from insectivorous bat because insect bat use echolocation characteristics to feed and navigate and also as one of the communicative function (Voight *et al.* 2010). They occupied several areas that serve abundance of food such as oil palm plantations, mangroves, orchards and primary rainforest. Fruit bat can be found to roost at different habitat such as foliage, cave, rock crevices, hollow trees and dead bamboos (Rahman *et al.*, 2011).

Frugivorous bats consumed various types of fruit and they play a vital role in pollination, as they become a seed disperser from the fruit they consumed. Size of the fruit can be varies and the fruit pulp can range between soft and hard (Freeman, 1988) but, they would not consume the entire food. Fruit bat crushes the food with their teeth and extracts the

juice from the food they chewed (Marshall, 1983). Fukuda *et al.* (2009) found that megachiropteran species ingest small seed and transported it over long distance and larger seed will be carried along to their feeding roost. The ability of bats as a dispersal agent helps in forest regeneration where healthy forest can reemerge (Kasso and Balakrishnan, 2013)

Frugivorous bats also benefit to agricultural industry as their guano has been used widely as natural fertilizer (Kasso and Balakrishnan, 2013). Countries like Thailand, Mexico, Jamaica and Indonesian has been practicing collecting and harvesting bat guano as it can increase their economic value (Wacharapluesadee, 2013). In Mexico, they had been extracting more than 10,000 tons of bat guano as agricultural fertilizer and shipped to fruit growers in California (Ducummon, 2000).

It is believes that food availability do influence bat fauna abundance and diversity in particular places (Robert *et al.*, 2004). Due to the temporal variation, migration of megachiroptera occurred where the exact local populations of fruit bat species changes over times such as in subtropical Australia (Robert *et al.*, 2004). The migration of bats from one area to another may associate with the distinction of microbial community present in the gut. This is because microbes in the gut need to evolve in order to adapt with the new environment in term of the food resources present in particular area. For example, *Bacillus thuringiensis* which commonly found in the gut of herbivorous animals inhabited in farms was found in the *Cynopterus brachyotis* gastrointestinal tract which detected occur due to the contamination of food that the *C. brachyotis* feed on the farm (Daniel *et al.*, 2013).

2.2 Microbe diversity

Microorganism or microbe is an organism that is microscopic and naked that plays an important role in host health and metabolism (Gill *et al.*, 2006; Taylor *et al.*, 2007; Ley *et al.*, 2008; Turnbaugh *et al.*, 2008 as cited in Barott, 2011). Microbe can be classified into five major groups which are fungi, bacteria, virus, protozoa and algae. The actual scale of life of microorganisms is unknown but because it is a very challenging task to estimates the diversity of life especially subjects that cannot be seen by naked eye (Patrick and Jo, 2004).

Microbes live in very close association with human. They can live symbiotically within mammalian gut, on the skin, in the soil, water and foods. Some of the bacteria can survive in very extreme environment such as Thermophilic bacteria. Thermophilic bacteria or thermophiles can only show visible growth with temperature more than 40 °C to 45 °C (Bergey, 1919). However, most of the bacteria can grow best at neutral pH and optimum temperature of 37 °C (Chiller *et al.,* 2001). Bacteria that commonly live within human environment are *Staphylococcus aureus*, *Streptococcus pharyngitis* and *Escherichia coli.*

Mammalians' gastrointestinal tract are colonised by a complex ecosystem with a countless of bacterial life inside (Mazmanian *et al.*, 2005). Bacteria are one of the life forms that exist on the earth about 3.8 billion ago and they have survived longer than human (Lisa, 2013). The colonisation of bacteria in organism's life starts right after birth and continues to increase in number as the organisms grow. However, it is yet to be explored on the number of species richness of bacteria in mammalian gut due to a vast number of microbial cells resides. In addition, they are not easily differentiated morphologically.

2.3 Bat gut microbiota

Gut microbiota is a composition of microbial population that lives in the mammalian gastrointestinal tract, which can provide benefit to the host or deteriorate host fitness (Sekirov *et al.*, 2010). Study on the microbial association in the bat gut is scarce and this knowledge is important to determine the ecological significance of this microbe to the host (Daniel *et al.*, 2013). There are various species of microbe in the bat gastrointestinal tract, which differed from one species to the other. These microbial community aids in various physiological aspect and important in the process of host biology such as development of host intestinal epithelium and improvement of immune system (Linnerbrink *et al.*, 2013). Previous study has shown that microbial community in the bat gut is host specific where they influence the nutritional status, behavior and host stress response (Sekirov *et al.*, 2010).

Microbial community in the mammalian gut shaped by a few aspects includes dietary intake and geographical regions. Ley *et al.* (2008) in his study claimed that various sources of food intake in mammalian diet have affected their gut microbial ecology. Bat gut microbiota may undergo evolution in a lifetime to meet the requirements in natural selection. Physical characteristics of fruit such as the size, odor, ripening of fruit and the hardness and softness of fruit skin affect the selection of fruit in foraging activity (Freeman, 1988).

Bacteria use host metabolism to grow and there are certain environment in the host that permit the growth of the bacteria. The physiological features include pH, available nutrition and chemical properties in the gut. There are two most common bacteria in the gastrointestinal tract of *Cynopterus brachyotis* which are the bacteria from the family *Enterobacteriaceae* and *Staphylococcaceae* (Daniel *et al.*, 2013). These microbes were

isolated and characterised by using molecular method and biochemical tests (Daniel *et al.*, 2013).

Previous studies on the intestine of bats recorded diverse species of bacteria that facilitate digestion of food in the gut of bats. Bacteria that colonize gastrointestinal tract of bat were differed from each species of bats such as insectivorous bats, frugivorous bats and nectar feeding bats. This is because, the diversity and abundance of bacteria in the gut affected by host diet (Ley *et al.*, 2008). For example, *Proteus vulgaris, Proteus mirabilis* and *Citrobacter freundii* were successfully isolated from the intestine of Indian flying fox, *Pteropus giganteus* that were believe to facilitate the digestion of xylan and cellulose in their diet (Anand and Sripathi, 2004). However, identification and characterisation of bat gut microbiota is a challenging area of study due to the limited reference for comparisons.

2.4 Biochemical test for bacterial identification

Biochemical test is a useful method in identification of unknown bacteria. Identification based on phenotypic characteristics such as color, shape, size always followed by the utilization of differential and selective media to grouped the unknown bacteria in to one family, genus or species (Bullock and Aslanzadeh, 2013). Biochemical tests comprised several tests which aid in differentiating biochemical activities possessed by each bacterium (Pujari, 2015). Different combination of growth test and enzyme test has been developed to provide more accurate and effective tests.

Bacterial identification by using biochemical tests is important because it provide productive method based on bacterial biochemical activities. Each species bacteria have developed specific characteristics of metabolic activities which is unique and different from one another. These unique characteristics not only help to identify bacteria but

characterize them according to their metabolic activities since identification based on morphological characteristics is influence by environmental factors and give inaccurate result.

Common biochemical test used in laboratory include citrate utilization test, bile esculin test, oxidase test, catalase test, triple sugar ion test and Voges-Proskauer test. Some of the biochemical tests were specifically developed to differentiate specific genus of bacteria. For example, bile esculin test was introduced to differentiate between members of *Streptococci* group D and *Enterococcus* (Acumedia, 2011). Family *Enterobacteriaciae* can easily be recognized based on a few similar characteristics which are gram negative bacilli, oxidase negative and mostly are catalase positive and motile by the presence of peritrichous flagella (UK standards for Microbiology investigations, 2015). However, the use of biochemical tests will not be able accurate result to differentiate between species of bacteria.

2.5 16S rRNA gene sequencing

Gene sequencing is a method to read the DNA sequence of an organism. Gene sequencing targeting at 16S ribosomal RNA is one of the significance methods in bacterial identification. 16S ribosomal RNA gene sequences analysis is a method to study the taxonomy and phylogeny of bacteria (Wang and Qian, 2009; Woo *et al.*, 2008). Gene sequence analysis using 16S rRNA gene has been widely used to identify an unknown bacteria up to species level. 16S rRNA is being used extensively because these genes universally present in all bacteria (Janda and Abbott, 2007; Calrridge, 2004). Thus, the relationships can be measured among all bacteria. Presence of the conserved region in this gene has makes it a good candidate for bacterial identification because it can be applied to

various organism. Conserved region is a region where the base sequence remains unchanged throughout evolution, which means that they evolved slowly. Furthermore, 16S rRNA has been pointed out to act as a molecular chronometer because of the highly conserved region present (Woose, 1987 as cited in Clarridge, 2004).

However, nowadays most of the researchers relied on metagenomics studies to solve advances activities of microorganism in terms of microbial ecology, evolution and diversity (Thomas *et al.*, 2012). Metagenomics is a branch of genomic study which focuses on the direct analysis of uncultured microorganism (Handelsman, 2004). The outcome from metagenomics study provides additional information regarding the microbial diversity on the earth as it measures 16S rRNA gene sequences directly from the environment (Handelsman, 2004). A study conducted by Hamady and Knight (2009) on metagenome of human has brought an insight on the biology of human microbiome and their role in human health and disease. Hence, metagenomic is a high-throughput analysis which led to more findings of new lineages of microbial life (Handelsman, 2004).

3.0 Materials and Methods

3.1 Material

List of materials is shown in Appendix 1.0.

3.2 Sampling sites

Different study sites in Sarawak have been chosen for collecting the bacterial samples from various species frugivorous bats. The sampling sites include Bako National Park and Samunsam Wildlife Sanctuary. Different study sites in term of the geographical area were chosen to compare the diversity of microbes in the gastrointestinal tract of frugivorous bats species from different locality. These selected sampling sites are mixed dipterocarp forest where it is a home for a lot of animals. These study sites were chosen because the availability or abundance of bat especially from frugivorous bats species. Figure 1.0 shows the locations of the study areas in Sarawak.

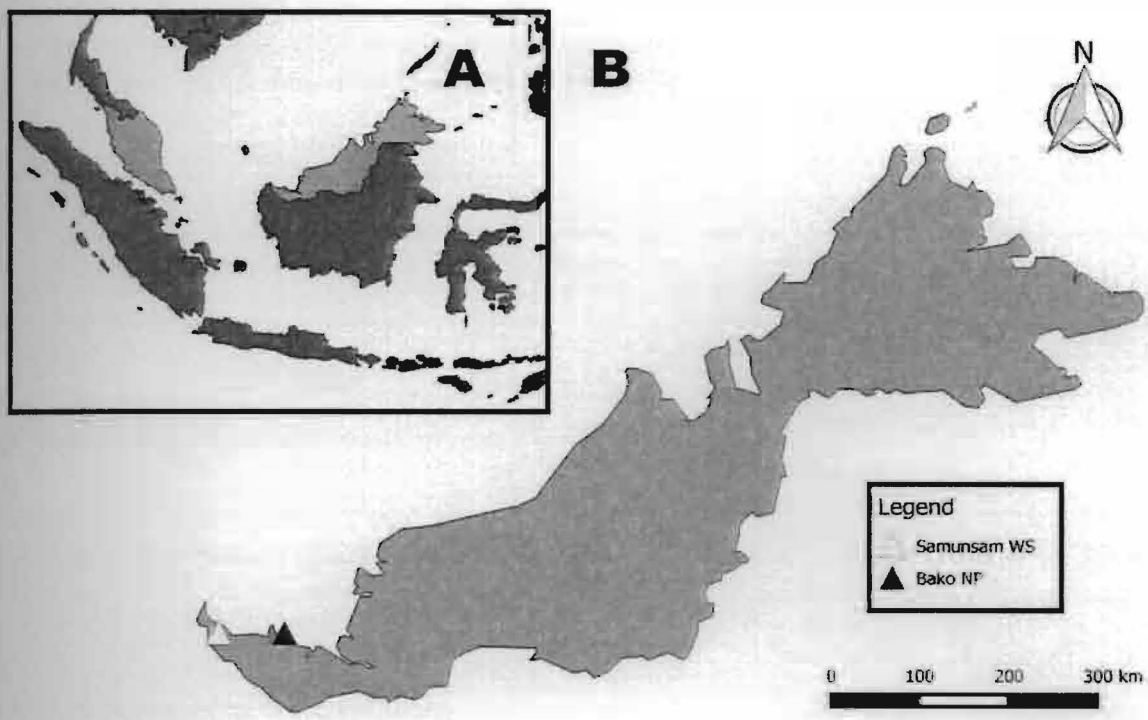

Figure 1.0. In set, map A is the region of Southeast Asia with Malaysia highlighted in grey color. Map B is the enlarge map of selected sampling sites.

3.3 Bat trapping and bat sample processing

Different species of frugivorous bats were captured by using mist nets. Mist nets were set in various locations that were detected to be the fly route of the bats. These mist nets were set from 1800 to 0700 hrs and the surveys were made twice per day; once in the evening (1800-2200) and once in the morning (0500-0700). The mist nets were observed for every 15 minutes in the first one hour on the evening because that is the most active time for the bats to go out for food. Fruit bats that have been trapped on the mist nets were untangled from the net and kept in the cloth bag (Figure 2.0). Live captured fruit bats were used to collect the bacterial sample. The genus and species of the fruit bats were identified according to Payne *et al.* (1985). Measurement of the ear, tail, forearm, wingspread, tragus and body length were taken as shown in Figure 2.0 by using Vernier caliper and weighed

by using Pesola spring balance (Payne *et al.*, 1985). Some of the location of the mist net was changed every day if there is no captured bat on the net. This is to make sure that enough samples of fruit bats were captured.

Figure 2.0. Pictures during bat trapping and sample processing: (A) Bat untangled from mist nest; (B) Bat measurement taken

3.4 Microbial sampling and processing

Anal swab method was used to isolate the bacterial samples from the anus of the fruit bat. To collect the bacterial samples, live captured frugivorous bats were chosen to obtain the fresh samples of the bacteria. The bacterial samples were used as a source of sample for biochemical and molecular study. Aseptic technique has been applied in this process to prevent contamination on the bacterial samples. Sterilized cotton bud was used to swab on the anus of the fruit bat. The cotton bud containing bacterial samples were soaked in the tube containing phosphate buffer saline (PBS) buffer pH 7.4 for the preservation purpose. The tubes were sealed properly to prevent contamination on the samples and labeled correctly according to the species of fruit bat used. Then, the tubes were kept in the ice box for preservation purposes.

3.5 Enumeration of bacteria

The bacterial samples that have been collected were processed by using enumeration method. Enumeration was done to determine the number of bacteria in the gastrointestinal tract of frugivorous bats captured. Total viable plate enumeration that involves serial dilutions and plate count agar technique was used to enumerate the bacterial samples (Harisha, 2005). Serial dilutions for each bacterial sample were performed from 10^{-1} to 10^{-7} dilutions. 100 µl of bacterial samples was pipetted into the tube containing 900 µl of PBS buffer. Vortex was used to make sure that the sample mix thoroughly with the buffer. Next, spread plate was done where 100 µl of dilution from each tube was taken and spread on the plate. Serial dilutions and spread plate method were done in triplicate number to make estimation on the total number of bacterial cell present on the plate. Next, the plates containing the bacterial suspension were incubated in the incubator at 37 °C for 24 hrs. Subsequent to incubation, the number of bacterial colonies grow on the plate were counted and recorded. Plates that contain 30 to 300 colonies were used for the calculation of the colony forming unit (CFU) per ml. Next, for obtaining the pure culture bacteria, 6 to 10 bacterial colonies that grow differently from others in terms of their morphological characteristics were chosen for culture purpose on the LB broth. LB broth containing the single colony of bacteria was kept in the shaker at 37 °C for overnight. Cloudy appearance of the LB broth indicates the growth of the bacteria in the broth. The broth then was used for streaking on the LB agar and kept on the glycerol for biochemical test and molecular study. LB agar was kept in the incubator at 37 °C overnight and the glycerol was kept in the freezer at -20 °C for preservation.